

## Research article

**Activation of adherent vascular neutrophils in the lung during acute endotoxemia**Vasanthi R Sunil<sup>1</sup>, Agnieszka J Connor<sup>1</sup>, Peihong Zhou<sup>1</sup>, Marion K Gordon<sup>1</sup>, Jeffrey D Laskin<sup>2</sup> and Debra L Laskin<sup>1</sup><sup>1</sup>Department of Pharmacology and Toxicology, Rutgers University, 160 Frelinghuysen Road, Piscataway, New Jersey 08854-8020, USA<sup>2</sup>Environmental and Community Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey, USA**Correspondence:** Debra L Laskin - [laskin@ehsi.rutgers.edu](mailto:laskin@ehsi.rutgers.edu)

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**Abstract**

**Background:** Neutrophils constitute the first line of defense against invading microorganisms. Whereas these cells readily undergo apoptosis under homeostatic conditions, their survival is prolonged during inflammatory reactions and they become biochemically and functionally activated. In the present study, we analyzed the effects of acute endotoxemia on the response of a unique subpopulation of neutrophils tightly adhered to the lung vasculature.

**Methods:** Rats were treated with 5 mg/kg lipopolysaccharide (i.v.) to induce acute endotoxemia. Adherent neutrophils were isolated from the lung vasculature by collagenase digestion and sequential filtering. Agarose gel electrophoresis, RT-PCR, western blotting and electrophoretic mobility shift assays were used to evaluate neutrophil activity.

**Results:** Adherent vascular neutrophils isolated from endotoxemic animals exhibited decreased apoptosis when compared to cells from control animals. This was associated with a marked increase in expression of the anti-apoptotic protein, Mcl-1. Cells isolated 0.5–2 hours after endotoxin administration were more chemotactic than cells from control animals and expressed increased tumor necrosis factor- $\alpha$  and cyclooxygenase-2 mRNA and protein, demonstrating that they are functionally activated. Endotoxin treatment of the animals also induced p38 and p44/42 mitogen activated protein kinases in the adherent lung neutrophils, as well as nuclear binding activity of the transcription factors, NF- $\kappa$ B and cAMP response element binding protein.

**Conclusion:** These data demonstrate that adherent vascular lung neutrophils are highly responsive to endotoxin and that pathways regulating apoptosis and cellular activation are upregulated in these cells.

**Keywords:** MAP kinase, Mcl-1, neutrophils, NF- $\kappa$ B, survival**Introduction**

Endotoxin is a major component of the cell wall of Gram negative bacteria. It is normally cleared from the blood by macrophages of the reticuloendothelial system predominantly localized in the liver [1]. Acute endotoxemia, characterized by excessive levels of endotoxin in the blood, can lead to endothelial injury, hypotension, multi-organ failure and death [1]. Endotoxin-induced tissue injury is associat-

ed with an inflammatory response and an accumulation of phagocytes at sites of damage. Neutrophils are major cellular components of an acute inflammatory reaction. These cells are rapidly recruited to areas of injury where they become activated to release reactive oxygen and nitrogen intermediates, cytokines, and lipid mediators, which aid in antigen destruction, but may also contribute to tissue injury [2].

Neutrophils are relatively short-lived cells removed from the body under homeostatic conditions by the process of apoptosis. To exert their biological effects during inflammatory reactions, neutrophils must remain at the injured site for prolonged periods of time. In this regard, recent studies have shown that inflammatory mediators such as lipopolysaccharide (LPS), granulocyte macrophage-colony stimulating factor and leukotriene B<sub>4</sub> delay apoptosis and prolong neutrophil survival in culture [3,4]. Neutrophils migrating into rodent lungs in response to LPS challenge have also been reported to exhibit delayed rates of apoptosis [5,6]. Similarly, increases in cytokine and growth factor activity, and reduced neutrophil apoptosis, have been described in various inflammatory lung diseases in humans including chronic obstructive pulmonary disease, acute respiratory distress syndrome and pneumonia [3,7,8].

In the present study, we characterized the effects of acute endotoxemia on the response of a unique population of neutrophils, which we have previously demonstrated are tightly adhered to the lung vasculature [9]. Because of their location within the tissue, these cells are likely to be more relevant than circulating neutrophils, or neutrophils recovered by bronchoalveolar lavage (BAL), to the pathophysiology of endotoxin induced lung injury. We found that administering endotoxin to rats resulted in an increased number of functionally active adherent vascular lung neutrophils. Moreover, these cells exhibited decreased apoptosis and expressed the anti-apoptotic protein, Mcl-1. This was correlated with the induction of total mitogen activated protein (MAP) kinases, as well as NF- $\kappa$ B and cAMP response element binding (CREB) protein transcription factor activity in the cells. These findings suggest a potential mechanism mediating decreased apoptosis and increased functional activation of adherent vascular neutrophils in the lung during acute endotoxemia.

## Materials and methods

### Animals and treatments

Female specific pathogen-free Sprague Dawley rats (200–225 g, 6–8 weeks) were purchased from Taconic (Germantown, NY, USA). Animals were housed in microisolator cages and were maintained on sterile food and pyrogen-free water *ad libitum*. Acute endotoxemia was induced by i.v. injection of rats with 5 mg/kg *Escherichia coli* LPS (serotype 0128:B12, Sigma Chemical Co., St. Louis, MO, USA). Data from control animals treated with PBS were pooled. Each experiment used one to two animals per treatment group and was repeated at least three times.

### Cell isolation

Rats were euthanized by i.p. injection of Nembutal (125 mg/kg) 0.5–48 hours after administration of endotoxin or control. Adherent vascular lung neutrophils were isolated from rats as previously described [10]. Briefly, the lung was

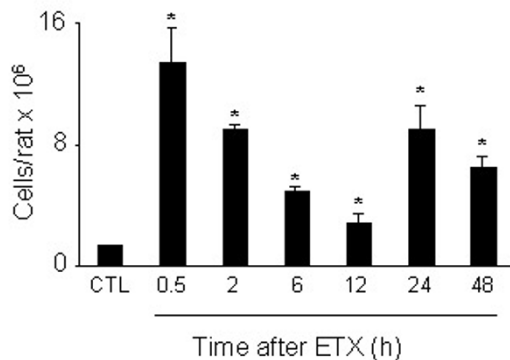
perfused with 50 ml of warm (37°C) Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Hank's balanced salt solution (HBSS, pH 7.4) containing 2.5 mM HEPES, 0.5 mM EGTA and 4.4 mM NaHCO<sub>3</sub> at a rate of 22 ml/min and then lavaged 5–6 times with HBSS to remove alveolar macrophages and loosely adhered neutrophils. The trachea and major bronchi were then excised and the lung cut into 500  $\mu$ m slices (Mclwain mechanical tissue chopper, Brinkmann Instruments, Westbury, NY, USA). Lung slices were washed in ice cold Ca<sup>2+</sup>/Mg<sup>2+</sup>-free HBSS with vigorous shaking at speed 7 for 3 min (Genie 2; Fisher Scientific, Pittsburgh, PA, USA), filtered (220  $\mu$ m), and then incubated in ice cold HBSS for 30 min with periodic shaking. Neutrophils were recovered, after digestion of the lung tissue for 25 min with 60 U/ml collagenase D (specific activity 0.22 Wunsch unit = 800 IU, low ancillary protease activity, Boehringer Mannheim, Indianapolis, IN, USA), prepared in HBSS containing 10% fetal bovine serum (FBS) and 0.01% DNase 1 (Sigma Chemical Co., St. Louis, MO, USA), followed by filtering (220  $\mu$ m). In previous studies using transmission electron microscopy, we demonstrated that cells recovered using this methodology consisted of a homogeneous population of neutrophils derived from the lung vasculature [9]. Giemsa staining of cytopspin samples showed that the cells were 96% neutrophils. Viability was greater than 90% as determined by trypan blue dye exclusion.

### Cytoplasmic DNA isolation and analysis

Cells ( $1 \times 10^6$ ) were lysed in 200  $\mu$ l of buffer (5 mM Tris-Cl pH 7.4, 2 mM EDTA, 0.5% Triton X-100) on ice under DNase-free conditions. After 30 min, cell lysates were centrifuged (16,500  $\times$  g for 20 min) and cytoplasmic DNA extracted from the supernatants by overnight precipitation in 0.1 $\times$  volume 3 M sodium acetate (pH 8.0) and 2 $\times$  volume 100% ethanol. After washing, DNA was dissolved in 10  $\mu$ l TBE buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0) [11]. Samples (5  $\mu$ l) were analyzed on 1.2% agarose gels, stained with ethidium bromide and visualized under UV light.

### Annexin V binding assay

Annexin V binding was detected using an Apoptosis Detection Kit (R & D Systems, Minneapolis, MN, USA). Adherent vascular lung neutrophils isolated from control animals, or 6–12 hours after administration of endotoxin, were plated for 18 hours into 4-well plates ( $5 \times 10^5$  cells/well). Cells were collected, washed and resuspended in PBS. Cells (100  $\mu$ l) were incubated in the dark in the presence of fluorescein-conjugated annexin V (10  $\mu$ l) and propidium iodide (10  $\mu$ l) reagent for 20 min at room temperature. Unstained cells, cells stained with annexin V fluorescein only, and cells stained with propidium iodide only, were used as additional controls. After diluting with PBS, cells were immediately analyzed on a Coulter Epics Profile II flow cytometer (Coulter Electronics, Hialeah, FL, USA).

**Figure 1**

Increased numbers of adherent vascular neutrophils in the lung following acute endotoxemia. Cells were isolated from control animals or 0.5–48 hours after endotoxin administration and enumerated microscopically. Each bar is the mean  $\pm$  SE ( $n = 4-9$ ). \*Significantly different ( $p < 0.01$ ) from control animals (ANOVA). CTL = control

#### Measurement of chemotaxis

Chemotactic responsiveness of lung neutrophils towards bacterially-derived *n*-formyl-methionyl-leucyl-phenylalanine (fMLP) was quantified using a Neuroprobe 48-microwell chemotaxis chamber and 5- $\mu$ m pore size Nucleopore filters [12,13]. Cells ( $2 \times 10^6$  cells/ml), suspended in HBSS containing 0.5% BSA, were incubated in the upper wells of the chamber at 37°C with fMLP (50 nM) or medium in the lower wells. After 45 min, the filter containing the adhered, migrated neutrophils was removed and stained with Camco Quik Stain (Baxter, McGaw Park, IL, USA). Chemotaxis was measured as the number of cells that migrated through the filter in 10 oil immersion fields.

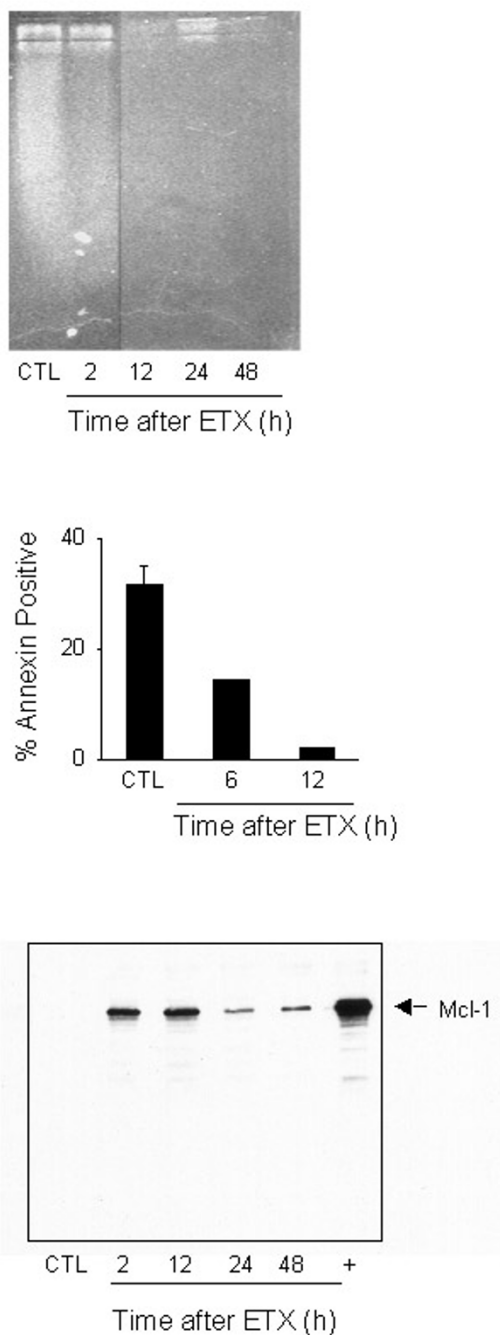
#### Relative RT-PCR

Total RNA was isolated from cells using an RNeasy Mini-prep kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. RNA was quantified by absorbance at 260 nm. For first strand synthesis, RNA (200 ng) in 9  $\mu$ l water was denatured at 65°C for 4 min, rapidly cooled on ice, and then resuspended in a 20  $\mu$ l final volume containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 1 mM of each dNTP, 20 mM random hexamers and 200 U Superscript II RNase H<sup>-</sup> RT (GIBCO BRL, Gaithersburg, MD, USA). After 1 hour incubation at 37°C, RNase H<sup>-</sup> (2 U) was added and the samples incubated for an additional 20 min. The samples were then denatured at 95°C for 5 min and chilled on ice. Initially, the linear range of amplification for cyclooxygenase-2 (COX-2) and tumor necrosis factor alpha (TNF- $\alpha$ ) mRNA was determined using mouse COX-2 and TNF- $\alpha$  primers (Ambion

Inc., Austin, TX, USA) with the neutrophil first strand cDNA samples as templates, following the manufacturer's instructions. The primers (5' or 3') used for COX-2 were sense primer, CAT TCT TTG CCC AGC ACT TCA C and anti-sense primer, GAC CAG GCA CCA AGA CCA AAG AC. For TNF- $\alpha$  the primers were sense primer, TCT GTC TAC TGA ACT TCG GGG T and antisense primer, TAG TTG GTT GTC TTT GAG ATC C. For COX-2 and TNF- $\alpha$ , 23 cycles and 26 cycles, respectively, fell within the linear range for each control and experimental sample. The 18S primer: competitor (Ambion Inc., Austin, TX, USA) ratio was adjusted to produce an amplification signal in the linear range of the COX-2 and TNF- $\alpha$  products, to normalize each COX-2 and TNF- $\alpha$ . A ratio of 1:19 was found to be appropriate to normalize both messages. Lung neutrophil cDNA was then amplified in 20  $\mu$ l buffer containing 1  $\mu$ l neutrophil reverse transcribed cDNA (representing 10 ng total RNA), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.4  $\mu$ M TNF- $\alpha$  primer or COX-2 primer, 1:19 ratio of 18S competitors/primers, 0.1 mM each dNTP, 0.2  $\mu$ l  $\alpha$  [<sup>32</sup>P] dCTP (10 mCi/ml; > 3000 Ci/mmol) and 0.025 units of AmpliTaq (Perkin Elmer Inc., Norwalk, CT, USA). Amplification was initiated by 1 min denaturation at 94°C, followed by 23 or 26 cycles (for COX-2 and TNF- $\alpha$  respectively) at 94°C for 15 sec, 56°C for 25 sec, and 72°C for 90 sec. The amplified PCR products (20  $\mu$ l total) were mixed with 10  $\mu$ l of loading buffer (95% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) and 5  $\mu$ l aliquots were applied to a 5% denaturing polyacrylamide gel. Radioactive bands were excised from the dried gel and counted by the Cerenkov method in a scintillation counter. Amplifications for all samples were performed at the same time and run on the same gel to minimize variability.

#### Western blot analysis

Cells were suspended in buffer containing 50 mM HEPES pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 1  $\mu$ g/ml pepstatin A, 1  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml soybean trypsin inhibitor, 10  $\mu$ g/ml aprotinin and 0.5% NP-40 and mixed periodically using a vortex. After 10 min on ice, lysates were centrifuged (4000  $\times$  g, 5 min) and supernatants containing 10  $\mu$ g of protein fractionated on 10% SDS polyacrylamide gels. The proteins were transferred to nitrocellulose paper, and incubated at room temperature for 3 hours, or overnight, at 4°C, with mouse monoclonal anti-human Mcl-1 (1:1000), p38 MAP kinase, phosphoinositide 3 kinase (PI3K), or protein kinase B- $\alpha$  (PKB- $\alpha$ ) (1:500) antibodies (Transduction Laboratories, San Diego, CA, USA and New England Biolabs, Inc., Beverly, MA, USA), or rabbit polyclonal anti-rat p44/42 MAP kinase (1:1000), or doubly phosphorylated anti-human Thr180/Tyr182 phospho-p38 MAP kinase (1:1000) antibodies (New England Biolabs, Inc.). According to the manufacturers, these antibodies cross react with rat cells. This was followed by incubation with a 1:2000 dilution of goat anti-mouse or sheep anti-rab-

**Figure 2**

Effects of acute endotoxemia on DNA fragmentation, Annexin V binding and Mcl-1 expression in adherent vascular lung neutrophils. Cells were isolated from control animals or 2–48 hours after endotoxin administration. *Upper Panel:* Cytoplasmic DNA was extracted, analyzed on 1.2 % agarose gels and visualized after staining with ethidium bromide. *Mid-panel:* Cells were analyzed for Annexin V binding by flow cytometry. Data are expressed as % Annexin V positive cells. *Lower Panel:* Cytoplasmic extracts were prepared and 10 µg protein/well analyzed for Mcl-1 protein by western blotting. Positive control (RAW 264.7 mouse macrophage lysates) is indicated by the + symbol. One representative gel of three separate experiments is shown. CTL = control

bit IgG horse-radish peroxidase-conjugated antibody (Transduction Laboratories and New England Biolabs) for 1 hour at room temperature. Proteins were detected using an Enhanced Chemi-Luminescence (ECL) detection system (Amersham Life Sciences, Arlington Heights, IL, USA). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) with BSA as the standard.

#### **Preparation of nuclear extracts and electrophoretic mobility shift assay**

Nuclear extracts were prepared as previously described [14]. Briefly, cells were incubated in buffer (10 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 2 mM EDTA) on ice for 10 min followed by incubation with 10% NP-40. After 5 min, cells were centrifuged (16,500 × g, 5 min), the pellets resuspended in buffer (50 mM HEPES pH 7.4, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol) and then placed on ice for an additional 20 min. Supernatants containing nuclear extracts were collected after centrifugation (16,500 × g, 5 min, 4°C). Binding reactions were carried out at room temperature for 30 min in a total volume of 15 µl and contained 2–5 µg of nuclear extracts, 5 µl of 5X gel shift binding buffer (20% glycerol, 5 mM MgCl<sub>2</sub>, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5), 2 µg poly (dI-dC) and 3 × 10<sup>4</sup> cpm/µl of [<sup>32</sup>P] labeled CREB (5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3'), NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3') (Promega Gel Shift Assay Systems, Madison, WI, USA) or CCAAT/enhancer binding protein (C/EBP) (5'-TGC AGA TTG CGC AAT CTG CA-3') (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) oligonucleotides. Probes were labeled using γ [<sup>32</sup>P]ATP (3000 Ci/mmol, NEN, Boston, MA, USA). Protein-DNA complexes were separated on 5% or 7% non-denaturing polyacrylamide gels run at 250 V in 0.5× TBE and visualized after the gels were dried and autoradiographed. For supershift reactions, extracts were incubated with antibodies (1 µg) to NF-κB subunits (p50 or p65), CREB, phospho-CREB, C/EBP-β or C/EBP-δ for 20 min on ice prior to the addition of labeled oligonucleotide. For competitor reactions, an excess (40×) of the respective unlabeled oligonucleotide was added to the reaction mixtures prior to addition of the labeled probe.

## **Results**

### **Effects of endotoxin on adherent vascular lung neutrophil number and survival**

Approximately 1 × 10<sup>6</sup> adherent vascular neutrophils were recovered from the lungs of control animals (Fig. 1). Treatment of rats with endotoxin caused a time-related increase in cell number. This response was rapid, occurring within 0.5 hours of endotoxin administration, and biphasic. Thus, the initial increase in cell number was followed by a decrease towards control levels and then a secondary increase at 24 hours, which persisted for 48 hours.

To determine if neutrophils responding to endotoxin administration exhibited reduced apoptosis, we analyzed the cells by DNA agarose gel electrophoresis. Freshly isolated neutrophils from control animals displayed significant apoptosis as indicated by the appearance of cytoplasmic DNA in the gels (Fig. 2, upper panel). Treatment of rats with endotoxin caused a time related decrease in cytoplasmic DNA, which was evident within 2 hours and became undetectable by 12 hours. The effects of *in vivo* endotoxin on neutrophil apoptosis persisted for at least 48 hours. We also analyzed apoptosis in adherent vascular lung neutrophils using Annexin V binding. Cells isolated from control animals showed significant apoptosis as demonstrated by increased Annexin V binding (Fig. 2, middle panel). Treatment of rats with endotoxin caused a time dependent decrease in Annexin V binding by adherent vascular lung neutrophils.

#### **Endotoxin administration induces Mcl-1 expression in adherent vascular lung neutrophils**

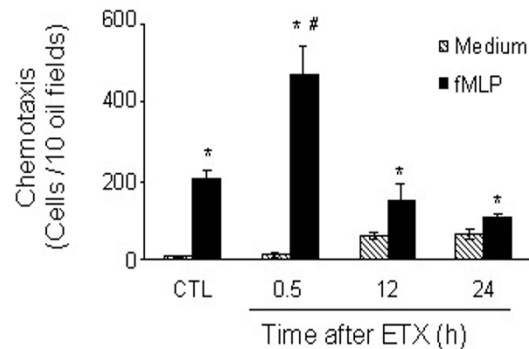
Mcl-1 is an anti-apoptotic member of the Bcl-2 family whose expression is correlated with decreased apoptosis and increased survival in human neutrophils [15,16]. Western blot analysis showed that neutrophils from control animals did not express Mcl-1 protein (Fig. 2, lower panel). Treatment of rats with endotoxin resulted in a rapid induction of Mcl-1, which peaked between 2 and 12 hours. Although expression of Mcl-1 in the neutrophils declined with time, significant amounts were still detectable 48 hours after endotoxin administration. In contrast, we were unable to detect Bcl-2, Bax or Bcl-X<sub>L</sub> in adherent vascular lung neutrophils even after endotoxin administration (data not shown).

#### **Effects of endotoxin on functional activity of adherent vascular lung neutrophils**

To determine whether acute endotoxemia activated adherent vascular lung neutrophils for increased functional responsiveness, we initially analyzed their chemotactic activity towards bacterially-derived fMLP. This peptide was found to be a potent chemoattractant for adherent vascular lung neutrophils (Fig. 3). Induction of acute endotoxemia resulted in a dramatic increase in their responsiveness to fMLP, which was evident in cells isolated 0.5 hours after treatment of the animals (Fig. 3). However, the effects of endotoxin were transient. Thus, by 12 hours post endotoxin administration, chemotactic responsiveness was at or below control levels.

Previous studies have demonstrated that acute endotoxemia activates peripheral blood neutrophils to release prostaglandins [17]. These lipid mediators are produced during inflammatory reactions largely via the enzyme COX-2 [18]. We found that COX-2 mRNA was markedly upregulated (40–400 fold) in adherent vascular lung neutrophils follow-

**Figure 3**



Effects of acute endotoxemia on chemotaxis. Cells were isolated from control animals or 0.5–24 hours after endotoxin administration. Chemotaxis was measured using microwell chambers. Data are the mean  $\pm$  SEM of three experiments. \*Significantly different ( $p \leq 0.05$ ) from medium control. #Significantly different ( $p \leq 0.05$ ) from CTL animals (ANOVA). CTL = control

ing endotoxin treatment of the animals (Fig. 4). This was evident within 0.5 hours of endotoxin administration and persisted for at least 12 hours. Subsequently, COX-2 mRNA returned to control levels. Induction of COX-2 mRNA following endotoxin administration was correlated with expression of COX-2 protein. However, in contrast to mRNA, protein levels remained elevated for 48 hours. Acute endotoxemia also resulted in a time dependent increase in TNF- $\alpha$  mRNA levels in adherent vascular lung neutrophils which was evident within 0.5 hours (Fig. 5). As observed with COX-2 mRNA, TNF- $\alpha$  mRNA levels began to decline at 12 hours and by 24 hours were at control levels.

#### **Effects of endotoxin on biochemical activity of adherent vascular lung neutrophils**

We next examined biochemical signaling pathways known to regulate cell survival and activation to determine if their expression was altered in adherent vascular lung neutrophils during acute endotoxemia. Initially, we analyzed total p38 and p44/42 MAP kinases. Freshly isolated cells from untreated animals expressed low levels of both total p38 and p44/42 MAP kinase as determined by western blot analysis (Fig. 6, upper panel). Induction of acute endotoxemia caused a rapid (within 0.5 hours) and persistent increase in expression of both proteins. Activation of MAP kinases is associated with their phosphorylation [19]. Neutrophils from control animals were found to express phospho-p38 MAP kinase. Endotoxin treatment of the animals was associated with a transient decrease in expression of activated p38 MAP kinase (Fig. 6, upper panel). In contrast to phospho-p38 MAP kinase, we were unable to detect

phospho-p44/42 MAP kinase in adherent vascular neutrophils (data not shown).

The effects of acute endotoxemia on expression of PI3K and its downstream target PKB- $\alpha$  were also investigated. Adherent lung neutrophils from control animals expressed low levels of PI3K (Fig. 6, lower panel). In contrast, PKB- $\alpha$  was not detectable in these cells. Administration of endotoxin to the animals induced PI3K expression, which peaked 12 hours post-treatment and persisted for 48 hours. This was correlated with induction of PKB- $\alpha$ . However, in contrast to PI3K, the effects of endotoxin on PKB- $\alpha$  were transient. Thus, PKB- $\alpha$  levels began to decline after 12 hours (Fig. 6, lower panel).

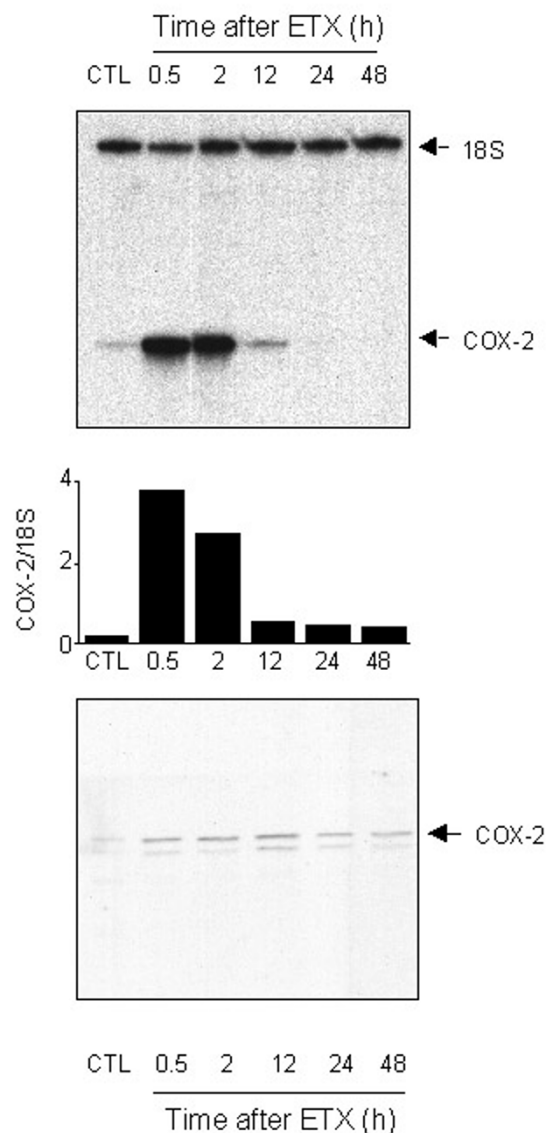
NF- $\kappa$ B, CREB and C/EBP are transcription factors known to be important in regulating the expression of various genes controlling apoptosis and inflammatory mediator production including Mcl-1, COX-2 and TNF- $\alpha$  [20,21], and these were next examined. No NF- $\kappa$ B nuclear binding activity was detected in cells from control animals (Fig. 7, left panel). Endotoxin administration caused a biphasic increase in NF- $\kappa$ B binding activity, which was most pronounced 0.5 hours and 24 hours post-treatment of the animals. Two major complexes were formed. Antibodies to p50 and p65 completely blocked the migration of the slower moving complex in the gel and partially blocked the migration of the faster moving complex. Binding was also abolished in the presence of excess unlabeled NF- $\kappa$ B, demonstrating the specificity of the probe.

As observed with NF- $\kappa$ B, constitutive CREB and C/EBP nuclear binding activities were not evident in cells from control animals (Fig. 7, middle and right panels). Although endotoxin administration caused a significant induction of both of these transcription factors in adherent vascular neutrophils, the kinetics of their responses was distinct. Thus, whereas CREB activity peaked 0.5 hours after endotoxin administration, C/EBP binding activity was maximal at 12 hours. For both transcription factors, binding was abolished by excess unlabeled probe, demonstrating specificity. Moreover antibodies to CREB, as well as phospho-CREB, blocked the nuclear binding activity of CREB, while antibodies to C/EBP- $\beta$  and C/EBP- $\delta$  blocked C/EBP nuclear binding activity.

## Discussion

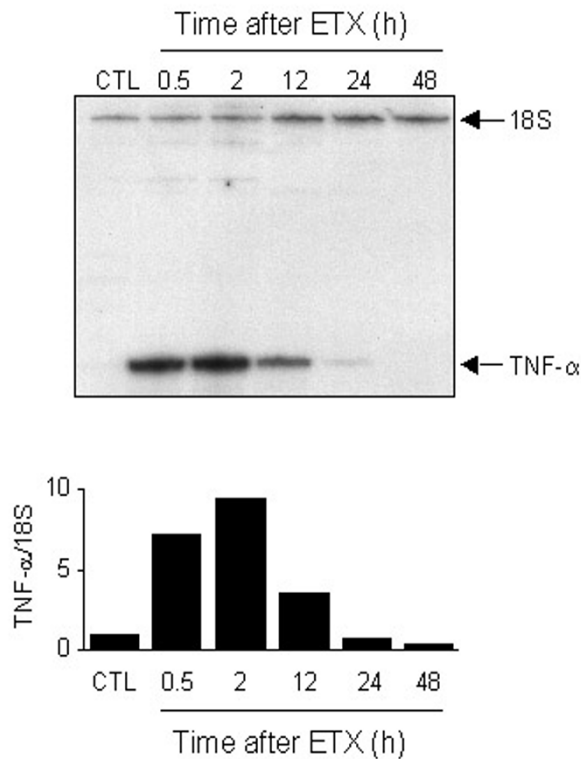
Recent studies have suggested that phagocytic leukocytes including macrophages and neutrophils contribute to the pathophysiology of endotoxin-induced lung injury [22]. Whereas macrophages are normally present in the tissue, the majority of neutrophils responding to endotoxin must emigrate into the lung from the blood. The initial step in this process involves tight adherence of the neutrophils to the vasculature [23]. In this study, we analyzed the effects of

**Figure 4**



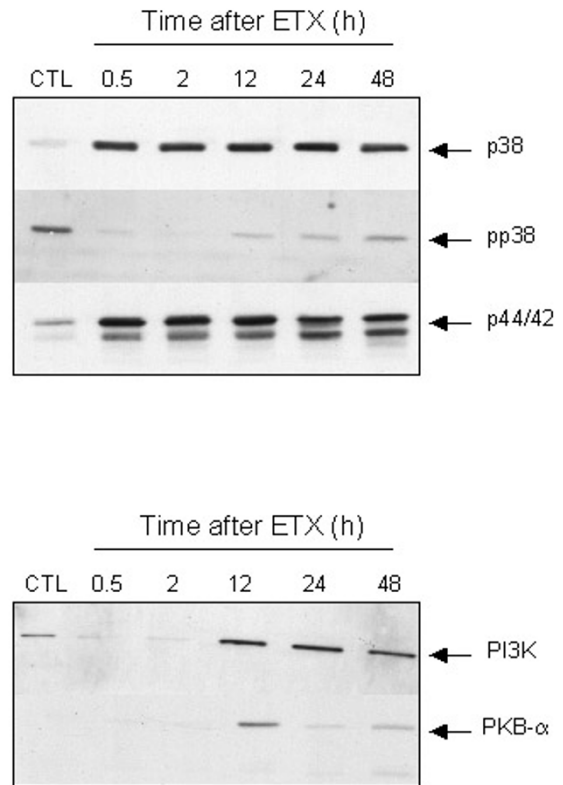
Induction of COX-2 mRNA and protein in adherent vascular lung neutrophils following endotoxin administration. Cells were isolated from control animals or 0.5–48 hours after endotoxin administration. *Upper panel:* Samples were amplified using specific COX-2 primers and run on denaturing polyacrylamide gels. *Middle panel:* Radioactivity of each amplified COX-2 cDNA band was normalized relative to its corresponding 18S band. *Lower panel:* Cytoplasmic extracts were analyzed for COX-2 protein expression by western blotting. One representative gel of three separate samples is shown. COX-2 = cyclooxygenase-2; CTL = control

acute endotoxemia on functional and biochemical responsiveness of these cells.

**Figure 5**

Effects of acute endotoxemia on TNF- $\alpha$  mRNA expression in lung neutrophils. Cells were isolated from control animals or 0.5–48 hours after endotoxin administration. *Upper panel*: Samples were amplified using specific TNF- $\alpha$  primers and run on denaturing polyacrylamide gels. *Lower panel*: Radioactivity of each amplified TNF- $\alpha$  cDNA band was normalized relative to its corresponding 18S band. CTL = control; TNF- $\alpha$  = tumor necrosis factor alpha

Treatment of rats with endotoxin was found to stimulate neutrophil adherence to the lung vasculature. Thus, following endotoxin administration, increased numbers of adherent vascular neutrophils were recovered from the lung. A similar increase in vascular lung neutrophils has been observed in rabbits following i.v. administration of LPS [24,25]. Interestingly in our model, this response was biphasic occurring initially at 0.5 hours and then again at 24 hours after endotoxin administration. We speculate that this represents two distinct populations of responding cells. Sepsis is associated with a rapid release of CXC chemokines by alveolar macrophages, as well as up regulation of lung vascular intercellular adhesion molecule-1 (ICAM-1) expression [26] and these may contribute to the initial increase in adherent vascular neutrophils in the lung. Activated neutrophils have also been reported to express increased macrophage inflammatory protein-2 (MIP-2) mRNA following endotoxin administration [21]. It is possible that the secondary increase in adherent neutrophils in

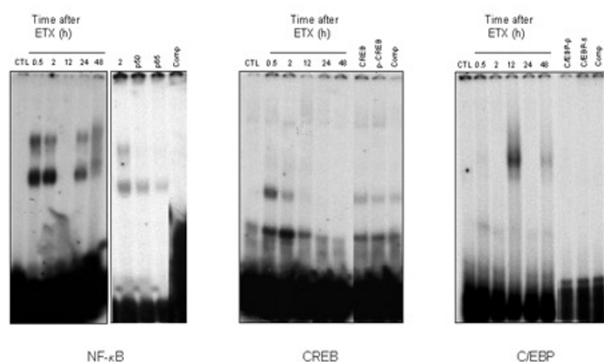
**Figure 6**

Effects of acute endotoxemia on MAP kinase, PI3K and PKB- $\alpha$  expression. Cells were isolated from control animals or 0.5–48 hours after endotoxin administration. Cytoplasmic extracts were prepared and 10  $\mu$ g protein/well analyzed for p38, phospho-p38 and p44/42 MAP kinase, PI3K and PKB- $\alpha$  expression by western blotting. One representative gel of three separate experiments is shown. CTL = control MAP = mitogen activated protein; PI3K = phosphatidylinositol 3 kinase; PKB = protein kinase B

the lung vasculature is due to the generation of chemotactic factors by activated neutrophils.

Mature neutrophils have a circulating life span *in vivo* of about 6–10 hours, after which time they undergo apoptosis [27]. The present studies demonstrate that adherent vascular neutrophils from the lungs of endotoxin treated animals exhibit decreased apoptosis as evidenced by reduced cytoplasmic DNA and decreased Annexin V binding when compared to cells from untreated animals. These results are in accord with previous studies demonstrating delayed apoptosis in neutrophils recovered from BAL fluid and from peripheral blood after LPS challenge in mice and humans [5,28] and correlate with our findings of increased survival of these cells in culture [29].

Acute endotoxemia caused a marked induction of Mcl-1 in freshly isolated adherent vascular lung neutrophils, which peaked between 2 and 12 hours and remained elevated for

**Figure 7**

Kinetics of NF- $\kappa$ B, CREB and C/EBP nuclear binding activity in adherent vascular lung neutrophils after endotoxin administration to rats. Cells were isolated from control animals or 0.5–48 hours after endotoxin administration. Nuclear extracts (2–5  $\mu$ g) were analyzed for NF- $\kappa$ B (left panel), CREB (middle panel) or C/EBP (right panel) binding activity. For supershift assays, extracts of cells isolated from rats 2 hours (NF- $\kappa$ B), 0.5 hours (CREB) or 12 hours (C/EBP) after endotoxin administration were pre-incubated with the indicated antibodies. To confirm the specificity of the oligonucleotides, the extracts were pre-incubated for 20 min with an excess of unlabeled NF- $\kappa$ B, CREB and C/EBP competitors (Comp). One representative gel of three separate experiments is presented. C/EBP = CCAAT/enhancer binding protein; CREB = cAMP response element binding protein; CTL = control; NF- $\kappa$ B = nuclear factor-kappa B

at least 48 hours. These findings indicate that prolonged neutrophil survival in the lung vasculature following endotoxin administration may be mediated, at least in part, by Mcl-1. In contrast to cells from endotoxin treated animals, adherent vascular neutrophils from control animals did not express Mcl-1 protein. This is consistent with the relatively high levels of apoptosis observed in these cells and their reduced survival in culture [29]. Our findings are in accord with a recent study demonstrating that Mcl-1 expression in human peripheral blood neutrophils decreases with aging [15]. As previously reported for human peripheral blood neutrophils [30], we were unable to detect Bcl-2 in rat adherent vascular lung neutrophils, even after endotoxin treatment of animals. We were also unable to detect Bax or Bcl-X<sub>L</sub> in rat adherent vascular lung neutrophils, which is in contrast to results with human peripheral blood neutrophils [30,31]. These differences may be due to unique attributes of human and rat cells, their distinct location in the vasculature, and/or to their exposure to inflammatory stimuli *in vivo*.

We have previously shown that acute endotoxemia primes adherent lung neutrophils for increased production of reactive oxygen and nitrogen intermediates suggesting that these cells are functionally activated [9]. The work reported here, demonstrates that adherent vascular lung neutrophils from endotoxin treated rats are also activated to respond to chemotactic stimuli and to express TNF- $\alpha$  and COX-2 mRNA and protein. The effects of endotoxin administration

on these responses were rapid, occurring within 0.5 hours. Subsequently, chemotactic responsiveness and TNF- $\alpha$  mRNA expression declined and by 12 hours were at control levels. Similar transient increases in neutrophil chemotaxis and pulmonary TNF- $\alpha$  production have been described previously during experimentally induced endotoxemia [32,33]. These findings are consistent with the idea that chemotaxis and TNF- $\alpha$  production are important in early inflammatory responses of neutrophils [34]. In contrast, protein levels for COX-2 remained elevated for 48 hours after endotoxin administration. This most likely reflects the sustained role of prostaglandins during the inflammatory process [35,36].

Inflammatory mediators induce their biological effects by binding to specific receptors on target cells. This initiates biochemical signaling pathways leading to increased survival and functional activation. Members of the MAP kinase signaling cascade have been shown to delay apoptosis in a number of cell types [37–39]. Proteins belonging to this family are also involved in cell signaling leading to functional responsiveness of inflammatory cells including chemotaxis and mediator production [40]. Treatment of rats with endotoxin induced expression of total p38 and p44/42 MAP kinase in adherent vascular lung neutrophils within 0.5 hours, a response which persisted for 48 hours. Our findings that these MAP kinases are upregulated within 0.5 hours in response to endotoxin suggests that they may contribute to increased chemotactic responsiveness and TNF- $\alpha$  production. In this regard, inhibition of p38 MAP kinase activity has been reported to block chemotaxis and TNF- $\alpha$  release by lung neutrophils, epithelial cells and eosinophils [40–42]. Interestingly, significant phospho-p38 MAP kinase was also detected in neutrophils from control animals. Phospho-p38 MAP kinase is known to promote apoptosis in human peripheral blood neutrophils [37] and a similar pathway may be involved in adherent vascular lung neutrophil apoptosis in control animals. Alternatively, endotoxin administration to animals may induce phosphatases that degrade phospho-p38 MAP kinase [43]. In contrast to phospho-p38 MAP kinase, we were unable to detect phospho-p44/42 MAP kinase in rat adherent vascular neutrophils even after endotoxin administration. This may be due to an inability of the antibody to recognize phosphorylated residues on these proteins in primary rat neutrophils. Abraham *et al* [44] have recently reported p42 MAP kinase activation in mouse lung neutrophils in response to endotoxin administration. Differences between our results may be due to unique attributes of rat and mouse models.

Inositol lipids generated via PI3K and downstream targets such as PKB- $\alpha$  have been implicated in the regulation of cell survival, as well as in induction of MAP kinases and TNF- $\alpha$  [45–47]. We found that endotoxin treatment of the animals caused a significant increase in expression of both



PI3K and PKB- $\alpha$  at 12 hours, which is consistent with reduced apoptosis in these cells. However our findings that PI3K levels remained elevated for 48 hours, while PKB- $\alpha$  decreased, suggest that PI3K regulates signaling pathways in adherent vascular neutrophils that are distinct from PKB- $\alpha$ . Our data also suggest that in lung neutrophils, PI3K and PKB- $\alpha$  are not co-ordinately regulated with chemotaxis, or expression of TNF- $\alpha$  or MAP kinases.

The transcription factors NF- $\kappa$ B and CREB have been reported to activate COX-2 in LPS-stimulated monocytes and macrophages *in vitro* [48,49]. Moreover, endotoxin-induced increases in neutrophil TNF- $\alpha$ , as well as interleukin-1 $\beta$  and MIP-2 *in vivo* appear to be mediated by NF- $\kappa$ B and CREB [21]. We found that activated NF- $\kappa$ B and CREB were present in the nucleus of adherent vascular lung neutrophils from endotoxemic, but not control rats, suggesting that these transcription factors play a role in increased responsiveness of the cells. These findings are in accord with previous studies on mouse lung neutrophils during endotoxemia [21]. Our observation that nuclear binding activity appeared in the cells rapidly after endotoxin administration (within 0.5 hours) indicates that NF- $\kappa$ B and CREB are involved in early functional activation. A similar role has been suggested for these transcription factors in the response of intraparenchymal mononuclear cells to hemorrhage [50]. The fact that NF- $\kappa$ B and CREB activation occurs prior to expression of PI3K and PKB- $\alpha$  indicates that these transcription factors may regulate these proteins in lung neutrophils. Interestingly, a secondary increase in NF- $\kappa$ B was observed in cells isolated 24 hours after endotoxin administration. This may reflect the activity of a second distinct neutrophil population responding to endotoxin.

Administration of endotoxin to the animals also induced C/EBP nuclear binding activity. In contrast to NF- $\kappa$ B and CREB, this was most pronounced 12 hours post-treatment of the animals. It is possible that NF- $\kappa$ B and CREB mediate the early phases of neutrophil activation, while C/EBP proteins function to maintain this response [50]. The observation that the temporal pattern of expression of activated C/EBP and PKB- $\alpha$  are similar suggests co-ordinated regulation of these proteins [51]. A decrease in activation of transcription factors like NF- $\kappa$ B and CREB and an increase in C/EBP may also contribute to reduced activity of pro-apoptotic genes, resulting in prolonged longevity of neutrophils in the lung vasculature. Recent studies have demonstrated upregulation of C/EBP in lung injury induced by LPS *in vivo* [52,53]. Our findings are consistent with this report and suggest that this transcription factor may be a marker of tissue injury.

## Conclusion

The present studies demonstrate that acute endotoxemia causes increases in the number of adherent neutrophils in

the lung vasculature. Moreover, these cells exhibit decreased apoptosis and increased chemotaxis and TNF- $\alpha$  expression, activities known to be important in early inflammatory responses. Acute endotoxemia also rapidly induced NF- $\kappa$ B and CREB, as well as total p38 and p44/42 MAP kinases. We speculate that NF- $\kappa$ B and CREB regulate the activity of biochemical signals leading to prolonged survival and inflammatory mediator production in the lung. Further studies are needed to determine the precise role of these signaling molecules in endotoxin-induced inflammatory responses in the lung.

## Abbreviations

BAL = bronchoalveolar lavage; BSA = bovine serum albumin; C/EBP = CCAAT/enhancer binding protein; COX-2 = cyclooxygenase-2; CREB = cAMP response element binding protein; FBS = fetal bovine serum; fMLP = n-formyl-methionyl-leucyl-phenylalanine; HBSS = Hank's balanced salt solution; ICAM-1 = intercellular adhesion molecule-1; LPS = lipopolysaccharide; MAP = mitogen activated protein; MIP-2 = macrophage inflammatory protein-2; NF- $\kappa$ B = nuclear factor-kappa B; PBS = phosphate-buffered saline; PI3K = phosphatidylinositol 3 kinase; PKB = protein kinase B; RT-PCR = Reverse transcription-polymerase chain reaction; TBE = Tris borate EDTA; TNF- $\alpha$  = tumor necrosis factor alpha

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